



APPLICATION No. 09/982,531

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## APPENDIX A

Claim 1 (Previously Presented) A method of producing a heterologous peptide, polypeptide or protein in a lactic acid bacterium, the method comprising the steps of

(i) constructing a recombinant lactic acid bacterium comprising a nucleotide sequence coding for the heterologous peptide, polypeptide or protein and operably linked thereto, appropriate regulatory nucleotide sequences to control the expression of the coding sequence,

(ii) cultivating said recombinant bacterium under fed-batch or continuous cultivation conditions in a chemically defined medium, to express the nucleotide sequence, and

(iii) harvesting the recombinant bacterium or the peptide, polypeptide or protein,

wherein the concentration of glucose is kept at a pre-selected concentration of at least about 0.5 g/L by controlled feeding of glucose.

Claim 2 (Previously Presented) A method according to claim 1 wherein the recombinant bacterium comprises a constitutive promoter operably linked to the coding sequence.

Claim 3 (Previously Presented) A method according to claim 1 wherein the recombinant bacterium comprises a regulatable promoter operably linked to the coding sequence.

Claim 4 (Previously Presented) A method according to claim 3 wherein the regulatable promoter is regulated by accumulation of a metabolite intracellularly or in the medium.

Claim 5 (Previously Presented) A method according to claim 3 wherein the regulatable promoter is derived from a lactic acid bacterium.

Claim 6 (Previously Presented) A method according to claim 5 wherein the regulatable promoter is the P170 promoter disclosed in WO 98/10079 or a derivative thereof.

Claim 7 (Previously Presented) A method according to claim 3 wherein the promoter is introduced into the lactic acid bacterium on an autonomously replicating replicon.

Claim 8 (Previously Presented) A method according to claim 3 wherein the promoter is a promoter not naturally associated with the nucleotide sequence coding for the heterologous peptide, polypeptide or protein.

Claim 9 (Original) A method according to claim 1 wherein the heterologous peptide, polypeptide or protein is selected from the group consisting of an enzyme and a pharmaceutically active compound.

Claim 10 (Original) A method according to claim 1 wherein the coding nucleotide sequence is operably linked to a nucleotide sequence coding for a signal peptide (SP).

Claim 11 (Original) A method according to claim 10 wherein the signal peptide is selected from the group consisting of the Usp45 signal peptide and the signal peptide having the sequence MKFNKKRVAIATFIALIFVSFFTISSQDAQAAERS (SEQ ID NO: 1).

Claims 12-13 (Cancelled)

Claim 14 (Previously Presented) A method according to claim 1 wherein the control of feeding of glucose to the medium is linked to pH control.

Claims 15-16 (Cancelled)

Claim 17 (Previously Presented) A method according to claim 1 wherein the yield of heterologous peptide, polypeptide or protein is at least 5 mg/L.

Claims 18-23 (Cancelled)

Claim 24 (Previously Presented) A method according to claim 1 wherein the chemically defined medium is the medium comprising:

Component	Concentration, mM or +/-
L-Alanine	3.4
L-Arginine	1.1
L-Asparagine	0.8
L-Cysteine	0.8
L-Glutamate	2.1
L-Glutamine	0.7

Glycine	2.7
L-Histidine	0.3
L-Isoleucine	0.8
L-Leucine	0.8
L-Lysine-HCl	1.4
L-Methionine	0.7
L-Phenylalanine	1.2
L-Proline	2.6
L-Serine	2.9
L-Threonine	1.7
L-Tryptophan	0.5
L-Tyrosine	0.3
L-Valine	0.9
K <sub>2</sub> SO <sub>4</sub>	0.28 <sup>a</sup>
KH <sub>2</sub> PO <sub>4</sub> /K <sub>2</sub> HPO <sub>4</sub>	4/6
Na-acetate	15
CaCl <sub>2</sub>	0.0005 <sup>a</sup>
MgCl <sub>2</sub>	0.52 <sup>a</sup>
FeSO <sub>4</sub>	0.01 <sup>a</sup>
Vitamins <sup>b</sup>	+
Micronutrients <sup>a,c</sup>	+
Citric acid	0.1

<sup>a</sup> From Neidhardt et al. J. Bacteriol. **119**:736-747;

<sup>b</sup> Vitamins: 0.4 µM biotin, 10 µM pyridoxal-HCl, 2.3 µM folic acid, 2.6 µM riboflavin, 8 µM niacinamide, 3 µM thiamine-HCl and 2 µM pantothenate;

<sup>c</sup> Micronutrients: 0.003 µM (NH<sub>4</sub>)<sub>6</sub>(MO<sub>7</sub>)<sub>24</sub>, 0.4 µM H<sub>3</sub>BO<sub>4</sub>, 0.03 µM CoCl<sub>2</sub>, 0.01 µM CuSO<sub>4</sub>, 0.08 µM MnCl<sub>2</sub> and 0.01 µM ZnSO<sub>4</sub>, or

wherein the components of said chemically defined medium are present in three-fold or five-fold amounts of the enumerated concentrations, except the phosphates and sodium acetate, the respective amounts of which are kept at the enumerated concentrations.

Claims 25-26 (Cancelled)

Claim 27 (Previously Presented) A method according to claim 1 wherein the chemically defined medium is the medium comprising:

Component	Concentration, mM or +/-
L-Alanine	3.4
L-Arginine	1.1
L-Asparagine	0.8
L-Cysteine	0.8
L-Glutamate	2.1

L-Glutamine	0.7
Glycine	2.7
L-Histidine	0.3
L-Isoleucine	0.8
L-Leucine	0.8
L-Lysine-HCl	1.4
L-Methionine	0.7
L-Phenylalanine	1.2
L-Proline	2.6
L-Serine	2.9
L-Threonine	1.7
L-Tryptophan	0.5
L-Tyrosine	0.3
L-Valine	0.9
K <sub>2</sub> SO <sub>4</sub>	0.28 <sup>a</sup>
KH <sub>2</sub> PO <sub>4</sub> /K <sub>2</sub> HPO <sub>4</sub>	4/6
Na-acetate	15
CaCl <sub>2</sub>	0.0005 <sup>a</sup>
MgCl <sub>2</sub>	0.52 <sup>a</sup>
FeSO <sub>4</sub>	0.01 <sup>a</sup>
Vitamins <sup>b</sup>	+
Micronutrients <sup>a,c</sup>	+
Citric acid	0.1

<sup>a</sup> From Neidhardt et al. J. Bacteriol. **119**:736-747;

<sup>b</sup> Vitamins: 0.4  $\mu$ M biotin, 10  $\mu$ M pyridoxal-HCl, 2.3  $\mu$ M folic acid, 2.6  $\mu$ M riboflavin, 8  $\mu$ M niacinamide, 3  $\mu$ M thiamine-HCl and 2  $\mu$ M pantothenate;

<sup>c</sup> Micronutrients: 0.003  $\mu$ M (NH<sub>4</sub>)<sub>6</sub>(MO<sub>7</sub>)<sub>24</sub>, 0.4  $\mu$ M H<sub>3</sub>BO<sub>4</sub>, 0.03  $\mu$ M CoCl<sub>2</sub>, 0.01  $\mu$ M CuSO<sub>4</sub>, 0.08  $\mu$ M MnCl<sub>2</sub> and 0.01  $\mu$ M ZnSO<sub>4</sub>;

wherein glucose is additionally included in the chemically defined medium in an amount in the range of 1-100 g/L, or

wherein glucose is additionally included in the chemically defined medium in an amount in the range of 1-100 g/L and wherein the components of said chemically defined medium are present in three-fold or five-fold amounts of the enumerated concentrations, except the phosphates and sodium acetate, the respective amounts of which are kept at the enumerated concentrations.

Claims 28-29 (Cancelled)

Claim 30 (New) A method of producing a heterologous peptide, polypeptide or protein in a lactic acid bacterium, the method comprising the steps of

- (i) constructing a recombinant lactic acid bacterium comprising a nucleotide sequence coding for the heterologous peptide, polypeptide or protein and operably linked thereto, appropriate regulatory nucleotide sequences to control the expression of the coding sequence,
- (ii) cultivating said recombinant bacterium under fed-batch or continuous cultivation conditions in a chemically defined medium supplemented with yeast extract, to express the nucleotide sequence, and
- (iii) harvesting the recombinant bacterium or the peptide, polypeptide or protein,

wherein the concentration of glucose is kept at a pre-selected concentration of at least about 0.5 g/L by controlled feeding of glucose.

Claim 31 (New) A method according to claim 30 wherein the recombinant bacterium comprises a constitutive promoter operably linked to the coding sequence.

Claim 32 (New) A method according to claim 30 wherein the recombinant bacterium comprises a regulatable promoter operably linked to the coding sequence.

Claim 33 (New) A method according to claim 32 wherein the regulatable promoter is regulated by accumulation of a metabolite intracellularly or in the medium.

Claim 34 (New) A method according to claim 32 wherein the regulatable promoter is derived from a lactic acid bacterium.

Claim 35 (New) A method according to claim 34 wherein the regulatable promoter is the P170 promoter disclosed in WO 98/10079 or a derivative thereof.

Claim 36 (New) A method according to claim 32 wherein the promoter is introduced into the lactic acid bacterium on an autonomously replicating replicon.

Claim 37 (New) A method according to claim 32 wherein the promoter is a promoter not naturally associated with the nucleotide sequence coding for the heterologous peptide, polypeptide or protein.

Claim 38 (New) A method according to claim 30 wherein the heterologous peptide, polypeptide or protein is selected from the group consisting of an enzyme and a pharmaceutically active compound.

Claim 39 (New) A method according to claim 30 wherein the coding nucleotide sequence is operably linked to a nucleotide sequence coding for a signal peptide (SP).

Claim 40 (New) A method according to claim 39 wherein the signal peptide is selected from the group consisting of the Usp45 signal peptide and the signal peptide having the sequence MKFNKKRVAIATFIALIFVSFFTISSQDAQAAERS (SEQ ID NO: 1).

Claim 41 (New) A method according to claim 30 wherein the control of feeding of glucose to the medium is linked to pH control.

Claim 42 (New) A method according to claim 30 wherein the amount of yeast extract is in the range of 0.1-10 g/L.

Claim 43 (New) A method according to claim 30 wherein the yield of heterologous peptide, polypeptide or protein is at least 5 mg/L.

Claim 44 (New) A method according to claim 30 wherein the chemically defined medium is the medium comprising:

Component	Concentration, mM or +/-
L-Alanine	3.4
L-Arginine	1.1
L-Asparagine	0.8
L-Cysteine	0.8
L-Glutamate	2.1
L-Glutamine	0.7
Glycine	2.7
L-Histidine	0.3
L-Isoleucine	0.8
L-Leucine	0.8
L-Lysine-HCl	1.4

L-Methionine	0.7
L-Phenylalanine	1.2
L-Proline	2.6
L-Serine	2.9
L-Threonine	1.7
L-Tryptophan	0.5
L-Tyrosine	0.3
L-Valine	0.9
K <sub>2</sub> SO <sub>4</sub>	0.28 <sup>a</sup>
KH <sub>2</sub> PO <sub>4</sub> /K <sub>2</sub> HPO <sub>4</sub>	4/6
Na-acetate	15
CaCl <sub>2</sub>	0.0005 <sup>a</sup>
MgCl <sub>2</sub>	0.52 <sup>a</sup>
FeSO <sub>4</sub>	0.01 <sup>a</sup>
Vitamins <sup>b</sup>	+
Micronutrients <sup>a,c</sup>	+
Citric acid	0.1

<sup>a</sup> From Neidhardt et al. J. Bacteriol. **119**:736-747;

<sup>b</sup> Vitamins: 0.4 µM biotin, 10 µM pyridoxal-HCl, 2.3 µM folic acid, 2.6 µM riboflavin, 8 µM niacinamide, 3 µM thiamine-HCl and 2 µM pantothenate;

<sup>c</sup> Micronutrients: 0.003 µM (NH<sub>4</sub>)<sub>6</sub>(MO<sub>7</sub>)<sub>24</sub>, 0.4 µM H<sub>3</sub>BO<sub>4</sub>, 0.03 µM CoCl<sub>2</sub>, 0.01 µM CuSO<sub>4</sub>, 0.08 µM MnCl<sub>2</sub> and 0.01 µM ZnSO<sub>4</sub>;

wherein the components of said chemically defined medium are present in three-fold or five-fold amounts of the enumerated concentrations, except the phosphates and sodium acetate, the respective amounts of which are kept at the enumerated concentrations.

Claim 45 (New) A method according to claim 30 wherein the chemically defined medium is the medium comprising:

Component	Concentration, mM or +/-
L-Alanine	3.4
L-Arginine	1.1
L-Asparagine	0.8
L-Cysteine	0.8
L-Glutamate	2.1
L-Glutamine	0.7
Glycine	2.7
L-Histidine	0.3
L-Isoleucine	0.8
L-Leucine	0.8

L-Lysine-HCl	1.4
L-Methionine	0.7
L-Phenylalanine	1.2
L-Proline	2.6
L-Serine	2.9
L-Threonine	1.7
L-Tryptophan	0.5
L-Tyrosine	0.3
L-Valine	0.9
K <sub>2</sub> SO <sub>4</sub>	0.28 <sup>a</sup>
KH <sub>2</sub> PO <sub>4</sub> /K <sub>2</sub> HPO <sub>4</sub>	4/6
Na-acetate	15
CaCl <sub>2</sub>	0.0005 <sup>a</sup>
MgCl <sub>2</sub>	0.52 <sup>a</sup>
FeSO <sub>4</sub>	0.01 <sup>a</sup>
Vitamins <sup>b</sup>	+
Micronutrients <sup>a,c</sup>	+
Citric acid	0.1

<sup>a</sup> From Neidhardt et al. J. Bacteriol. 119:736-747;

<sup>b</sup> Vitamins: 0.4 µM biotin, 10 µM pyridoxal-HCl, 2.3 µM folic acid, 2.6 µM riboflavin, 8 µM niacinamide, 3 µM thiamine-HCl and 2 µM pantothenate;

<sup>c</sup> Micronutrients: 0.003 µM (NH<sub>4</sub>)<sub>6</sub>(MO<sub>7</sub>)<sub>24</sub>, 0.4 µM H<sub>3</sub>BO<sub>4</sub>, 0.03 µM CoCl<sub>2</sub>, 0.01 µM CuSO<sub>4</sub>, 0.08 µM MnCl<sub>2</sub> and 0.01 µM ZnSO<sub>4</sub>;

wherein glucose is additionally included in the chemically defined medium in an amount in the range of 1-100 g/L, or

wherein glucose is additionally included in the chemically defined medium in an amount in the range of 1-100 g/L and wherein the components of said chemically defined medium are present in three-fold or five-fold amounts of the enumerated concentrations, except the phosphates and sodium acetate, the respective amounts of which are kept at the enumerated concentrations.